Rapid Identification of Pathogenic *Vibrio* Species by Multilocus PCR-Electrospray Ionization Mass Spectrometry and Its Application to Aquatic Environments of the Former Soviet Republic of Georgia

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The Ibis T5000 is a novel diagnostic platform that couples PCR and mass spectrometry. In this study, we developed an assay that can identify all known pathogenic Vibrio species and field-tested it using natural water samples from both freshwater lakes and the Georgian coastal zone of the Black Sea. Of the 278 total water samples screened, 9 different Vibrio species were detected, 114 (41%) samples were positive for V. cholerae, and 5 (0.8%) samples were positive for the cholera toxin A gene (ctxA). All ctxA-positive samples were from two freshwater lakes, and no ctxA-positive samples from any of the Black Sea sites were detected.

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The Ibis T5000 is a novel diagnostic platform that couples PCR and mass spectrometry. In this study, we developed an assay that can identify all known pathogenic *Vibrio* species and field-tested it using natural water samples from both freshwater lakes and the Georgian coastal zone of the Black Sea. Of the 278 total water samples screened, 9 different *Vibrio* species were detected, 114 (41%) samples were positive for *V. cholerae*, and 5 (0.8%) samples were positive for the cholera toxin A gene (*ctxA*). All *ctxA*-positive samples were from two freshwater lakes, and no *ctxA*-positive samples from any of the Black Sea sites were detected.

The genus *Vibrio*, within the family *Vibrionaceae*, is a diverse group of Gram-negative bacteria found exclusively in the aquatic environment. Important pathogenic members include *Vibrio cholerae*, the causative agent of cholera, and *Vibrio parahaemolyticus* and *Vibrio vulnificus*, which have been implicated in diarrhea, septicemia, and wound infections (5). The Ibis T5000 uses electrospray ionization-mass spectrometry to analyze the products of broad-range PCR (PCR-electrospray ionization-mass spectrometry [PCR/ESI-MS]) and is designed to rapidly detect and identify emerging pathogens and biothreat agents without prior knowledge of a pathogen's nucleic acid sequence (4, 10).

Forty-two well-characterized bacterial strains were used for the validation of the *Vibrio* PCR/ESI-MS assay (Table 1). Four sampling sites, the Chorokhi estuary, Bulvari, Green Cape, and the Supsa estuary along the Georgian coastal zone of the Black Sea, and three freshwater lakes, Kumisi Lake, Lisi Lake, and the Tbilisi Sea near the city of Tbilisi, Georgia (Fig. 1), were sampled monthly from July 2006 to October 2007 and biweekly during the summer (July to September). One hundred liters of surface water was collected and processed by following methods described previously (8).

An 8-primer-pair assay with breadth of coverage across the entire family *Vibrionaceae* was developed (see Fig. S1 in the supplemental material). All primers used in the assay are listed in Table 2. All PCRs were performed in 96-well microtiter plates as described previously (1). A Bruker Daltonics microTOF (Billerica, MA) mass spectrometer was used for analyzing the purified DNA as described previously (3, 7). The presence of *V. cholerae* was confirmed by using species-specific intergenic spacer region (ISR) PCR primers (2). For detection of the cholera toxin A gene (*ctxA*), PCR was performed as described by Rivera et al. (9). For confirmation of the *Vibrio* population data obtained with the PCR/ESI-MS assay, an environmental clone library was constructed from one of the samples (collection date, 12 August 2006) from the Chorokhi estuary using the universal 16S rRNA primer 27F (5′-AGAGTTTGATYMTGGCTCAG-3′) and the *Vibrio*-specific primer 680R (5′-GAAATTCT ACCCCCCTCTACAG-3′). The resulting PCR product was cloned and sequenced, and sequences with identities of ≥99% were considered matches.

Base composition data were produced for all 35 *Vibrio* isolates (20 strains of *V. cholerae* and 15 non-cholera *Vibrio* species) (Fig. 2). Based on unique DNA base composition signatures, all the *Vibrio* strains were correctly identified at the species level. Reactions were negative for *Photobacterium damselae* and for all species of *Aeromonas* tested (data not shown). To determine the ability of the assay to detect mixtures of multiple species of *Vibrio*, we spiked equal amounts of one to four different *Vibrio* species in phosphate-buffered saline. In all cases, the assay successfully identified the mixture components correctly (see Fig. S2 in the supplemental material).

The limits of detection for PCR/ESI-MS were tested using

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TABLE 1. Bacterial strains used in this study

Species and/or strain[a]	Description	Source (yr of isolation)[b]
Vibrio strains		
V. cholerae O1		
026	El Tor Ogawa *ctxAB*$^+$	Kenya (1985)
MJ-1236	El Tor Inaba *ctxAB*$^+$	Matlab, Bangladesh (1994)
B33	El Tor Ogawa *ctxAB*$^+$	Beira, Mozambique (2004)
BX 330286	El Tor Inaba *ctxAB*$^+$	Australia; water (1986)
12129(1)	El Tor Inaba *ctxAB* mutant	Australia; water (1985)
CIRS101	El Tor Inaba *ctxAB*$^+$	Dhaka, Bangladesh (2002)
098	Classical *ctxAB*$^+$	Indonesia (1991)
TM 11079-80	El Tor Ogawa *ctxAB* mutant	Brazil; sewage (1980)
FMU 87295/0	Classical *ctxAB*$^+$	Mexico; clinical isolate (1983)
UMSM N-16961	El Tor Inaba *ctxAB*$^+$	Bangladesh (1975)
O395	Classical Ogawa *ctxAB*$^+$	India (1965)
V. cholerae O139		
MO10	*ctxAB*$^+$	India (1992)
MO45	*ctxAB*$^+$	India (1985)
CO-393	*ctxAB*$^+$	India (1993)
EM0158	*ctxAB*$^+$	Mathbaria, Bangladesh; pond water (2004)
V. cholerae non-O1, non-O139		
TMA 21	*ctxAB* mutant	Brazil; seawater (1982)
TMA 135	*ctxAB* mutant	Brazil; seawater (1981)
CB98-179	*ctxAB* mutant	Chesapeake Bay, Maryland, USA; water
UM4056	*ctxAB* mutant	Mud Lake, Louisiana, USA; water
CT 5369-93	*ctxAB* mutant	Brazil; sewage (1993)
V. vulnificus		
324 (CDC B9629)		Florida, USA; clinical isolate, ATCC 27562
332 (CDC A6546)*		Alaska, USA; clinical isolate, ATCC 33816
V. parahaemolyticus		
EB 101		Japan; clinical isolate, type strain ATCC 17802
EB 102		Japan; clinical isolate, ATCC 17803 (1953)
V. mimicus		
VM223		
MB-451		Bangladesh
V. metschnikovii LMG 11664		Fowl; ATCC 700040
V. albensis VL426		Maidstone, Kent, UK; water
V. furnissii 9119-82		Japan; human feces, CIP 102972
V. hollisae		Maryland, USA; human feces, CIP 101886
V. logei 584		Gut of Arctic mussel; ATCC 29985
V. mediterranei 50		Spain; coastal sediment, CIP 103203
V. cincinnatiensis		Ohio, USA; human clinical samples, CIP 104173
V. coralliilyticus YB		Tanzania; diseased coral, ATCC BAA-450
V. orientalis 717		China; seawater, CIP 102891 (1983)
Other bacteria		
Aeromonas caviae ATCC 15468		ATCC
Aeromonas hydrophila ATCC 7966		ATCC
Aeromonas jandaei		Brazil (1996)
Aeromonas salmonicida subsp. *masoucida* ATCC 27013		ATCC
Aeromonas sobria ATCC 9071		ATCC
Aeromonas veronii ATCC 35624		ATCC
Photobacterium damselae subsp. *damselae* ATCC 33539		ATCC

[a] *, confirmed as *V. mimicus* by 16S rRNA sequence analysis and biochemical characterization.
[b] ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur.

serial dilutions of the following three distinct *Vibrio* species: *V. cholerae*, *V. vulnificus*, and *V. alginolyticus*. Twofold serial dilutions of purified DNA were made, with a final concentration ranging from 0.05 ng to 50 ng per reaction mixture. Each sample was diluted 1:100 with genome dilution buffer and analyzed by PCR/ESI-MS. To confirm reproducibility, samples were run in duplicate. All three test strains showed detection at approximately 4 to 8 genome copies per PCR (see Table S1 in the supplemental material).

To test the ability of the assay to detect and identify *Vibrio* spp. from natural aquatic samples, we applied it to a subset of total community DNA samples collected in 2006 from freshwater lakes and sites along the Georgian coast of the Black Sea. Six different *Vibrio* species were detected and identified from 19 natural water samples collected from both freshwater and seawater sites spanning the seasons summer (July/August) to winter (November/December) in 2006 (Table 3). There was a high prevalence of *V. cholerae* DNA detection

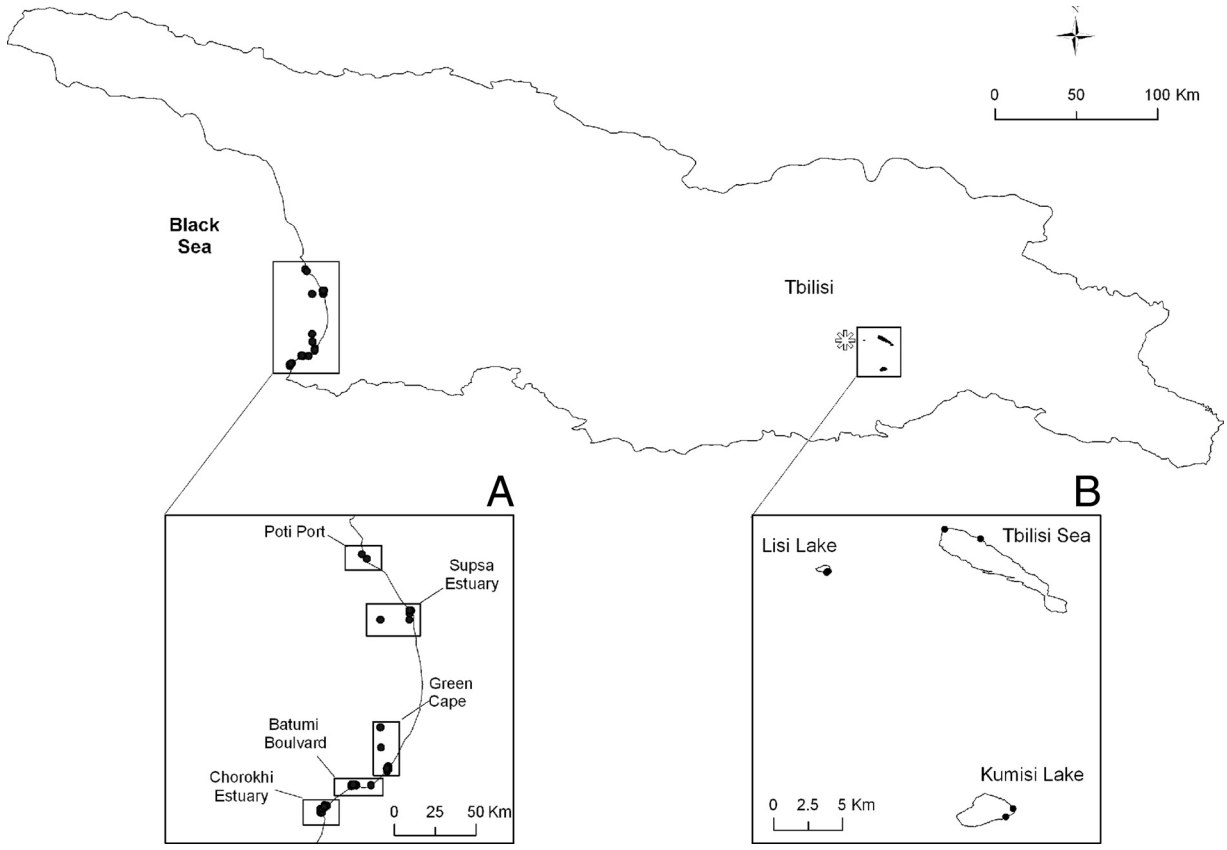

FIG. 1. Map of Georgia with sampling sites. (A) Georgian coast of the Black Sea showing the sites Poti, Supsa, Green Cape, and Batumi. (B) Map of the three freshwater lakes, with two sampling sites at each lake. (Adapted from reference 6 with permission of the publisher.)

for several of the sites in Georgia, evidenced by 13 out of the 19 samples testing positive for *V. cholerae*. All but one of these detections was confirmed by standard PCR targeting the *V. cholerae*-specific 16S-23S rRNA ISR. As expected, *Vibrio* counts, as determined by plate counting on plates with thiosulfate, citrate, bile salts, and sucrose (TCBS plates), were high in the summer months and decreased significantly during the winter months. For the Chorokhi estuary samples, counts fell from 3,500 CFU/100 ml in July to as low as 7 CFU/100 ml in December. Conversely, molecular analysis of the water samples using the PCR/ESI-MS assay suggested the presence of large numbers of vibrios late into the winter (Table 3), suggesting the possibility that these organisms were in the viable but nonculturable state.

TABLE 2. PCR primers used in the *Vibrio* PCR/ESI-MS assay

Primer pair no.	Orientation[a]	Sequence (5′–3′)	Target gene	Breadth of coverage
1098	F	TCCGCGGAGTTGACTGGGT	RNase P	All *Vibrionaceae*
	R	TGACTTTCCTCCCCCTTATCAGTCTCC		
2001	F	TGAGTGCCAACATATCAGTGCTGAAGA	*fur*	All *Vibrionaceae*
	R	TCCGCCTTCAAAATGGTGGCGAGT		
2011	F	TAAAGCCCGTGAAATGACTCGTCGTAAAGG	*gyrB*	All *Vibrionaceae*
	R	TGAGTCACCCTCCACAATGTATAGTTCAGA		
2993	F	TTCCCACCGATATCATGGCTTACCACGG	*ompU*	*Vibrio* and *Listonella*
	R	TCGGTCAGCAAAACGGTAGCTTGC		
2927	F	TCAATGAACGACCAACAAGTGATTGATG	*gapA*	All *Vibrionaceae*
	R	TCCTTTATGCAACTTGGTATCAACAGGAAT		
3003	F	TCAGCATATGCACATGGAACACCTC	*ctxB*	*V. cholerae* specific
	R	TGCCGTATACGAAAATATCTTATCATTTAGCGT		
2012	F	TACGCTGACGGAATCAACCAAAGCGG	*ompU*	*V. cholerae* specific
	R	TGCTTCAGCACGGCCACCAACTTCTAG		
2323	F	TGCCAAGAGGACAGAGTGAGTACTTTGA	*ctxA*	*V. cholerae* specific
	R	TAACAAATCCCGTCTGAGTTCCTCTTGCA		

[a] F, forward; R, reverse.

Species Name	Strain Name	2927-*gapA*	2001-*fur*	2993-*ompU*	2011-*gyrB*	1098-*RNAse P*	3003-*ctxB*	2323-*ctxA*	2012-*ompU*
V. albensis	VL426	[31 21 27 26]	[38 28 37 39]	[40 32 36 32]	[35 35 26 28]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[19 22 28 27]
V cholerae	EM0158	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V cholerae	CO-393	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V cholerae	UMSM N16961	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V. cholerae	CT 5369-93	[24 24 22 35]	[39 30 36 37]	[42 33 36 29]	[27 28 35 34]	[19 19 29 17]	[40 16 23 26]	[23 16 23 31]	[27 28 23 18]
V. cholerae	TM 11079-80	[24 24 22 35]	[37 37 29 39]	[35 34 34 37]	[35 34 27 28]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V. cholerae	O98	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V. cholerae	FMU 87295/0	[35 21 24 25]	[39 30 35 38]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	MO45	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V. cholerae	MO10	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[40 16 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	CB98-179	[24 24 22 35]	[39 30 36 37]	[37 33 35 35]	[28 28 35 33]	[20 18 28 18]	No Prime	No Prime	[28 27 22 19]
V. cholerae	TMA 21	[24 24 22 35]	[39 30 36 37]	[40 32 35 33]	[29 27 34 34]	[19 19 29 17]	No Prime	No Prime	No Prime
V. cholerae	TMA 135	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[33 35 27 29]	[19 19 29 17]	No Prime	No Prime	[28 27 22 19]
V. cholerae	O395	[35 21 24 25]	[39 30 35 38]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	MJ-1236	[33 20 24 28]	[42 34 26 40]	[44 27 37 32]	[24 31 32 37]	[31 23 43 23]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	B33	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	BX 330286	[24 24 22 35]	[37 37 29 39]	[39 32 36 33]	[28 28 34 34]	[20 18 29 17]	No Prime	No Prime	No Prime
V. cholerae	12129(1)	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	CIRS101	[24 24 22 35]	[41 34 26 41]	[41 32 36 31]	[37 32 29 26]	[28 22 41 29]	[41 15 22 27]	[23 16 23 31]	[28 27 22 19]
V. cholerae	O26	[24 24 22 35]	[39 30 36 37]	[39 32 37 32]	[33 35 27 29]	[19 19 29 17]	No Prime	No Prime	No Prime
V. cholerae	UM4056	[24 24 22 35]	[37 37 29 39]	[41 32 36 31]	[28 28 35 33]	[19 19 29 17]	[41 15 21 28]	[23 16 23 31]	[28 27 22 19]
V. cinncinnatiensis	CIP 104173	[31 21 27 26]	[40 27 36 39]	[48 28 35 29]	[29 26 37 32]	[20 17 29 18]	No Prime	No Prime	No Prime
V. coralliilyticus	BAA-450	No Prime	[38 27 35 42]	[39 27 40 34]	[25 29 33 37]	[22 16 30 16]	No Prime	No Prime	No Prime
V. furnissii	CIP 102972	[33 21 24 27]	[40 27 35 40]	No Prime	[34 26 32 32]	[37 20 43 20]	No Prime	No Prime	No Prime
V. hollisae	CIP 101886	[27 23 23 32]	[40 27 36 39]	[45 26 37 32]	[27 29 32 36]	[22 17 32 15]	No Prime	No Prime	No Prime
V. logei	584	[31 21 27 26]	[37 29 37 39]	[35 30 41 34]	[25 29 38 32]	[22 16 30 16]	No Prime	No Prime	No Prime
V. mediterranei	CIP 103203	[27 23 23 32]	[45 25 34 38]	[45 26 37 32]	[27 29 32 36]	[22 17 32 15]	No Prime	No Prime	No Prime
V. metschnikovii	LMG 11664	[33 19 27 26]	[38 29 37 38]	[46 29 38 27]	[28 29 32 35]	[20 18 29 17]	No Prime	No Prime	No Prime
V. mimicus	MB-451	[30 21 28 26]	[37 28 37 40]	[40 32 35 33]	[28 27 35 34]	[18 19 30 17]	No Prime	No Prime	No Prime
V. mimicus	VM223	[31 21 27 26]	[38 28 37 39]	[40 32 36 32]	[28 26 35 35]	[19 19 29 17]	No Prime	No Prime	No Prime
V. orientalis	717	[31 21 27 26]	[38 24 35 45]	[38 27 39 36]	[30 27 31 36]	[33 22 43 23]	No Prime	No Prime	No Prime
V. parahaemolyticus	EB 101	[33 20 25 27]	[40 26 34 42]	[45 26 37 32]	[24 31 32 37]	[31 23 43 23]	No Prime	No Prime	No Prime
V. parahaemolyticus	EB 102	[33 20 25 27]	[43 33 25 41]	[45 26 37 32]	[24 31 32 37]	[31 23 43 23]	No Prime	No Prime	No Prime
*V. vulnificus**	332	[30 22 28 25]	[37 29 37 39]	[40 32 34 34]	[27 28 35 34]	[19 19 29 17]	No Prime	No Prime	No Prime
V. vulnificus	324	[32 21 30 22]	[37 38 26 41]	[39 31 38 32]	[31 27 34 32]	[32 22 43 23]	No Prime	No Prime	No Prime

*Confirmed as *V. mimicus* by 16S sequencing

FIG. 2. Base compositions of eight selected amplicons from *Vibrio* ribosomal and housekeeping genes. Within each column, base compositions that are common to multiple types are similarly colored. The numbers in the columns indicate the numbers of each base (A, G, C, and T) in the PCR amplicons generated for each gene target. Base compositions with a white background are unique to a particular type. No Prime, no PCR amplification product was expected.

TABLE 3. PCR/ESI-MS detection and quantification of *Vibrio* species in selected Georgian natural water samples

Sampling site	Date of sampling (day-mo-yr)	Sample type	Total *Vibrio* sp. count (CFU/100 ml)	PCR/ESI-MS detection (no. of genome copies/reaction mixture)[a]	*V. cholerae*-specific ISR PCR[c]
Chorokhi	27-Jul-06	Enriched	3,500	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	Pos
Bulvari	27-Jul-06	Enriched	3,000	*V. parahaemolyticus*, *V. vulnificus*	ND
Chorokhi[b]	10-Aug-06	Enriched	1,320	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	ND
Chorokhi	27-Aug-06	Enriched	1,600	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	Pos
Supsa	27-Aug-06	Enriched	275	*V. cholerae*, *V. parahaemolyticus*, *V. vulnificus*	Pos
Kumisi Lake	6-Sept-06	Enriched		*V. cholerae*	Pos
Tbilisi Sea	9-Sept-06	Concentrated		*V. cholerae* (38)	Pos (weak)
Supsa	20-Sept-06	Enriched	300	*V. cholerae*, *V. vulnificus*	Pos
Green Cape	20-Sept-06	Enriched	1,400	*V. parahaemolyticus*, *V. vulnificus*	ND
Chorokhi	20-Sept-06	Enriched	400	*V. cholerae*, *V. parahaemolyticus*	Pos
Green Cape	25-Oct-06	Concentrated	200	*V. cholerae* (6,998)	Pos
Chorokhi	24-Oct-06	Concentrated	600	*V. vulnificus* (67)	ND
Tbilisi Sea	30-Oct-06	Concentrated		*V. cholerae* (5,583)	Pos
Lisi Lake	7-Nov-06	Enriched		*V. cholerae*	Pos
Tbilisi Lake	7-Nov-06	Concentrated		*V. cholerae* (3,266)	Pos
Lisi Lake	7-Nov-06	Enriched		*V. mimicus*	ND
Chorokhi	2-Dec-06	Concentrated	7	*V. alginolyticus* (172), *V. vulnificus* (178)	ND
Bulvari	2-Dec-06	Enriched	88	*V. metschnikovii*	ND
Supsa	2-Dec-06	Enriched	60	*V. cholerae*, *V. parahaemolyticus*	Neg

[a] Quantitation not shown for enriched samples.
[b] Chosen for 16S rRNA environmental clone library.
[c] ND, not done; Pos, positive; Neg, negative.

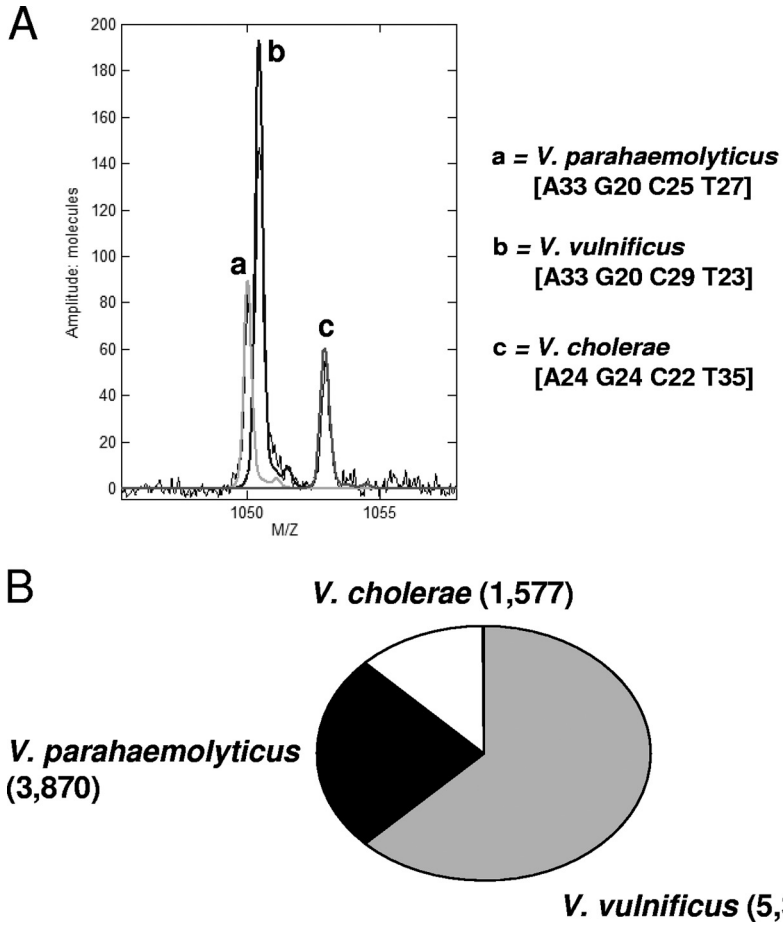

FIG. 3. Detection of multiple pathogenic *Vibrio* species in a sample collected in August 2006 from the Chorokhi estuary site on the coast of the Black Sea. (A) Mass spectra of PCR amplicons derived from the RNase P housekeeping gene from total community DNA extracted from the sample showing detections of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. (B) Relative proportion of DNA sequences matching *Vibrio* species obtained from an environmental 16S rRNA clone library using primers 24F and 680R, as described in Materials and Methods. For comparison, the genomic quantifications (numbers of genome copies per reaction mixture) derived from the PCR/ESI-MS assay are given in parentheses.

For further confirmation of the PCR/ESI-MS results, we constructed a 16S rRNA clone library for the Chorokhi estuary sample collected on 10 August 2006 (Fig. 3). *Vibrio* population analysis using the PCR/ESI-MS assay revealed the presence of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* in the sample (Fig. 3A). Sequence identification by clone library analysis revealed the presence of the same *Vibrio* species, and their relative abundances were similar to those indicated by the PCR/ESI-MS analysis (Fig. 3B).

For the 278 total water samples screened, 9 different *Vibrio* species were detected, with 114 (41%) samples positive for *V. cholerae* and 5 (0.8%) positive for the *ctxA* gene. All *ctxA*-positive samples were from two freshwater lakes, either Kumisi Lake or Lisi Lake, located near the capital city of Tbilisi, Georgia. No *ctxA*-positive samples were detected from any of the Black Sea sites. All *ctxA*-positive samples, except for one, were confirmed by using *ctxA*-specific standard PCR.

To determine the specificity of the assay to identify vibrios isolated from Georgian natural waters, we characterized 10 *Vibrio* isolates, comprising five different species isolated from both freshwater lakes and the Black Sea. All 10 isolates were correctly identified with high confidence using the PCR/ESI-MS assay (see Table S2 in the supplemental material). Importantly, the results show that *Vibrio mimicus* can be correctly identified and distinguished from the closely related species *V. cholerae*.

Our objectives in this study were to develop an assay based on PCR/ESI-MS using the Ibis T5000 platform for the rapid detection and identification of all pathogenic vibrios and then to field-test it using natural water. This study marks the first application of this technology to *Vibrio* spp. and the first use of the Ibis T5000 platform for the direct detection of bacterial pathogens from natural aquatic environments.

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